Diagnostic molecular oncology

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ABSTRACT

Objective: Molecular analysis based on fluorescence in-situ hybridization, southern blotting and the polymerase chain reaction, has in recent years emerged as a powerful tool to identify disease specific markers with diagnostic and prognostic significance. This manuscript concentrates on the polymerase chain reaction which is becoming the most widely used molecular technique in diagnostic pathology. Polymerase chain reaction detects the presence of a particular type of deoxyribonucleic acid fragment and can be performed on blood, body fluids, bone marrows, tissue biopsies and even formalin fixed paraffin embedded tissues. Technically, the polymerase chain reaction is highly sensitive, straight forward, and cost effective with a turn-around time shorter than other assays in molecular pathology.

Methods: The molecular diagnostics laboratory at King Faisal Specialist Hospital and Research Centre was established at the end of 1994 and for the last three years, more than 1400 samples from the oncology service has been analyzed for the detection of immunoglobulin heavy chain gene re-arrangement (IGH), T-cell receptor gene re-arrangement, BCR/ABL fusion gene for chronic myeloid leukemia, PML/RARα fusion gene for acute promyelocytic leukemia, and BCL-2 for follicular lymphoma.

Results: Using primers for IgH gene 80.3% of B-cell lymphoproliferative diseases were detected. T-cell clonality was detected in 91.7% of T-cell acute lymphoblastic leukemia. Bcl-2 gene re-arrangement was detected in 36% of follicular lymphoma cases. Using the RT-polymerase chain reaction, 9 cases of acute promyelocytic leukemia showed the presence of PML/RARα gene.

Conclusion: The application of polymerase chain reaction in diagnostic oncology has enormous potential for complementing morphology and flow cytometry and promises to serve as a deoxyribonucleic acid/ribonucleic acid marker to assist in the diagnosis, staging and monitoring of lymphoma and leukemia and in the detection of minimal residual disease after bone marrow transplantation.

Keywords: Molecular diagnostics, polymerase chain reaction, lymphoma, leukemia.


The rapid transition and application of molecular biologic techniques to diagnostic pathology has resulted in the availability of indispensable tools for a multitude of uses in the diagnostic laboratory. The fundamental feature of malignancies, that they are clonal, makes them particularly suitable for molecular-based diagnostic studies. While molecular assays are used in the evaluation of non-hematologic/solid tumors, current routine molecular diagnostic studies involve predominantly hematopoietic tumors and, in particular, malignant lymphoma and leukemia.

A variety of questions can be answered by the molecular analysis of lymphoproliferative processes: 1. establishment of the diagnosis of lymphoma in a neoplastic disorder of uncertain type (differential diagnosis of a lymphoid versus a nonlymphoid malignant neoplasm); 2. documentation of clonality (clonal or malignant versus polyclonal or reactive lymphoproliferative process; 3. assignment of
lineage (B-cell versus T-cell lymphoproliferative process); 4. demonstration of oncogene involvement associated with specific subtypes of lymphomas (bcl-1 in mantle cell lymphoma, bcl-2 in follicular center cell lymphoma, bcl-3 in chronic lymphocytic leukemia and bcl-6 in diffuse large cell lymphoma; 5. identification of oncogenes associated with disease progression (tumor progression genes - p53 and c-myc). 1-8

Molecular information regarding gene rearrangements and chromosomal translocations such as BCR/ABL, t(9;22) in chronic myeloid leukemia and PML/RAR-related translocations such as PML/RARA, t(15;17) in acute promyelocytic leukemia can be obtained from peripheral blood and bone marrow aspirates. Molecular work-up has been successfully performed on formalin fixed paraffin embedded tissues, allowing pathologists to perform retrospective analysis of archival specimens.

Methods. DNA extraction. High molecular weight DNA was isolated from fresh bone marrow aspiration, peripheral blood, paraffin embedded lymph node and skin biopsy specimens by standard proteinase K digestion and organic extraction procedures.9

Immunoglobulin gene rearrangement. Enzymatic amplification of the IgH gene was performed in a Perkin Elmer GeneAmp PCR System 9600 using a single V\textsubscript{H} primer homologous with a highly conserved sequence near the 3' end of FR3 in conjunction with a single consensus J\textsubscript{H} primer. Briefly, the reaction mixture contained 0.5 μg of DNA; 10 mM of Tris-HCl, pH 8.3; 200 μM of each dNTP; 50 mM KCl; 1.5 mM MgCl\textsubscript{2}; 10 pmol/L of each primer and 1 U Taq polymerase (Ampli-Taq; Perkin Elmer-Cetus, Norwalk, CT, U.S.A.) in a final volume of 25 μl. The consensus V\textsubscript{H} and J\textsubscript{H} primers were synthesized by Oligos, Etc. (Portland, Oregon, U.S.A.). The sequences were V\textsubscript{H} 5’ CTGAAATTCCAGGAGGGGA AGGCCCAACAG 3’; V\textsubscript{H} 5’CTCAATTCGGGAAATGCACTGAGC 3’; V\textsubscript{H} 5’CTGAAATTCGGGAAATGCACTGAGC 3’; V\textsubscript{H} 5’CTGAAATTCGGGAAATGCACTGAGC 3’; and Y\textsubscript{H} 5’CTGAGTCCACCTGTGA CAACAGTGTGTTC 3’.

The reaction mixture was subjected to 40 cycles of PCR following an initial 5 minute denaturation step at 94°C. Each cycle consisted of a 30 second denaturation step at 94°C, a 30 second annealing step at 62°C and a one minute elongation step at 72°C. The last cycle was followed by a nine minute elongation step at 72°C. Ten μl of PCR amplified product was resolved by electrophoresis on a 6% polyacrylamide gel, stained with ethidium bromide and visualized under ultraviolet light. A discrete band(s) is seen in clonal T-cell processes within the predicted size range of 190-260 base pairs.10 All samples were subjected to amplification using primers for the erb-B2 gene to confirm that amplifiable DNA was present.

BCL2 major breakpoint region. Enzymatic amplification of the BCL2 major breakpoint region rearrangement was performed in a Perkin Elmer GeneAmp PCR System 9600 using a oligonucleotide primer of a region 5’ to the BCL2 MBR and a single consensus J\textsubscript{H} primer. The 5’ BCL2 MBR primer was synthesized on a Applied Biosystem DNA Synthesizer and the J\textsubscript{H} primer was synthesized by Oligos, Etc. (Portland, Oregon, U.S.A.). The sequences were BCL2 MBR 5’ GAG AGT TGC TTT ACG TGG CCT G 3’12 and J\textsubscript{H} 5’ AAC TGC AGA GGA GAC GGT GAC C 3’. The reaction mixture contained 10 mM of Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl\textsubscript{2}; 1.001% w/v gelatin; 200 μM of each dNTP; 20 pmol/L of each primer; 1 U Taq polymerase (Ampli-Taq, Perkin Elmer-Cetus, Norwalk, CT, U.S.A.) and 1 μl of DNA digest in a final volume of 25 μl. The mixture was subject to 30 cycles of PCR following an initial 5 minute denaturation step at 94°C. Each cycle consisted of a 30 second denaturation step at 94°C, a 30 second
annealing step at 60°C and a 1 minute elongation step at 72°C. The last cycle was followed by a nine minute elongation step at 72°C. Ten microliters of PCR amplified product was resolved by electrophoresis on a 1.5% agarose gel and transferred to a nylon membrane. A discrete band of approximately 200 bp is expected for positive results. The membrane was hybridized at 55°C with the probe 5'CAA CAC AGA CCC ACC CAG AGC 3'. This probe was labelled for chemiluminescent detection using the ECL3' Labelling and Detection Kit (Amersham International, Buckinghamshire, England). In this labelling method, a fluorescein-11-dUTP tail is added to the probe's 3' end using terminal transferase. Following hybridization and stringency washes, the blots are incubated with anti-fluorescein-horseradish peroxidase conjugate. Excess antibody conjugate is removed by washing. The bound peroxidase labelled probe is detected with chemiluminescence detection reagents. When these two reagents are mixed together, the enzymatic reduction of peroxide is coupled to the oxidation of luminol. As the luminol breaks down, 428nm wavelength light is emitted and detected on X-Omat AR film (Eastman Kodak Company, Rochester, New York, USA).

**BCR/ABL.** Total cellular RNA was prepared from blood or bone marrow buffy coats and stored at -70°C until assayed. cDNA was synthesized using Promega's Reverse Transcription System (Madison, Wisconsin, USA). The 20μl reaction mixture contained 5mM MgCl2, 10mM Tris-HCl, 50mM KCl, 0.1% Triton X-100, 1mM each dNTP, 20U ribonuclease inhibitor, 15U AMV reverse transcriptase, 250ng oligo(dT) primer and 100-200 ng sample RNA in RNase-free water. The reaction mixture was incubated at 42°C for 15 minutes, then heated at 95°C for 5 minutes. The tubes were held briefly at 4°C until the PCR step. A 3' primer sequence for reverse transcription of BCR/abl is 5'-GGT ACC AGG AGT GTT TCT CCA GAC TG-3'. A PCR reaction mixture was prepared with the 5' primer sequence 5'-GAG CGT GCA GAG TGG AGG GAG AAC A-3'. After an initial 5 minute denaturation step at 94°C, PCR was performed in a Perkin Elmer GeneAmp PCR System 9600 by 30 cycles of 94°C 1 minute, 62°C 1 minute, 2 minutes followed by a 10 minute extension at 72°C. Ten microliters of amplified product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized in ultraviolet light. A band seen at either 430 or 500 base pairs is interpreted as positive. All samples were subjected to amplification using primers for the actin gene to confirm that mRNA was present. A band is always present at approximately 200 bp.

**PML-RARα.** Total cellular RNA was prepared from blood or bone marrow buffy coats and stored at 70°C until assayed. cDNA was synthesized using Promega's Reverse Transcription System (Madison, Wisconsin, U.S.A.). The 20μl reaction mixture contained 5mM MgCl2, 10mM Tris-HCl, 50mM KCl, 0.1% Triton X-100, 1mM each dNTP, 20U ribonuclease inhibitor, 15U AMV reverse transcriptase, 250ng oligo(dT) primer and 100-200 ng sample RNA in RNase-free water. The reaction mixture was incubated at 42°C for 15 minutes, then heated at 95°C for 5 minutes. The tubes were held briefly at 4°C until the PCR step. A PCR reaction mixture was prepared with the 3' primer sequence 5'-ACC GAT GGC TTC GAC GAG TTC-3' and the 5' primer sequence 5'-AGC CCT TGC AGC CCT CAC AG-3'. After an initial 5 minute denaturation step at 94°C, PCR was performed in a Perkin Elmer GeneAmp PCR System 9600 by 30 cycles of 94°C 1 minute, 55°C 1 minute, 72°C 1 minute followed by a 10 minute extension at 72°C. Ten microliters of amplified product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. A band seen at 220 base pairs or 550 and 694 base pairs is interpreted as positive. All samples were subjected to amplification using primers for the actin gene to confirm that mRNA was present.

**Results.** Immunoglobulin gene rearrangement. DNA from 127 specimens with confirmed B-cell lymphoproliferative disease by morphology and immunophenotyping were subjected to PCR amplification using primers for the framework 3 (FR3) sequence of VH. The different diagnostic categories and percentage of positivity obtained for IgH gene rearrangement are shown in Table 1.

<table>
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<tr>
<th>DIAGNOSIS</th>
<th>NUMBER OF SPECIMENS</th>
<th>PERCENT</th>
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<tbody>
<tr>
<td>B- Lineage ALL</td>
<td>65/74</td>
<td>87.7</td>
</tr>
<tr>
<td>Relapsed B-Lineage ALL</td>
<td>14/21</td>
<td>66.7</td>
</tr>
<tr>
<td>B-CLL</td>
<td>9/9</td>
<td>100.0</td>
</tr>
<tr>
<td>Multiple Myeloma</td>
<td>3/5</td>
<td>60.0</td>
</tr>
<tr>
<td>HCL</td>
<td>2/2</td>
<td>100.0</td>
</tr>
<tr>
<td>BCL-2 positive FL</td>
<td>3/7</td>
<td>43.0</td>
</tr>
<tr>
<td>B-Cell DLC lymphoma</td>
<td>4/6</td>
<td>66.7</td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>2/3</td>
<td>66.7</td>
</tr>
<tr>
<td>TOTAL</td>
<td>102/127</td>
<td>80.3</td>
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ALL: Acute Lymphoblastic Leukemia
CLL: Chronic Lymphocytic Leukemia
HCL: Hairy Cell Leukemia
FL: Follicular Lymphoma
DLC: Diffuse Large Cell Lymphoma
Discretely sized amplified products (one or two IgH-VDJ-PCR bands) ranging from 90-160 bp (Fig. 1), indicative of a clonal population with rearranged IgH gene, were evident in 102 of the 127 specimens with B-cell lymphoproliferative disease.

**T-cell receptor gene rearrangement.** Using three reaction mixtures for T-cell receptor gamma chain, a discrete band was visualized in one of the three runs representing the three reaction mixtures within the predicted size range of 190-260 base pairs in 22/24 (91.7%) cases of previously determined T-cell acute lymphoblastic leukemia (Fig. 2), and in 11/38 (28.9%) of B-cell lineage acute lymphoblastic leukemia positive for IgH-FR3 by PCR.

Twenty three cases of suspected cutaneous T-cell lymphoma (CTCL) were analyzed by PCR for the presence of T-cell receptor gene rearrangement utilizing DNA extracted from skin biopsies. Seventeen cases showed full concordance between morphologic examination and PCR results.

Three cases (13%) were false negative and three cases were positive in the absence of morphologic diagnosis of CTCL. Three months later, a follow-up biopsy demonstrated the presence of same molecular findings and full morphologic picture of CTCL in two cases out of three.

**BCR/ABL.** Fifty eight patients clinically presented with chronic myeloid leukemia (CML) were routinely studied by conventional cytogenetic and single pair primer RT-PCR techniques. A band seen at either 430 or 500 bp is interpreted as positive (Fig. 3). A comparison of predictive value is shown in Table 2. Cytogenetics predicted 90.6% and RT-PCR 96.8% of CML cases. Since January 1997, we have shifted into nested RT-PCR technique with

<table>
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<th>Table 2</th>
<th>Comparison of cytogenetics and single pair primer RT-PCR in CML.</th>
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<tr>
<td>No. of Cases</td>
<td>Cytogenetics</td>
</tr>
<tr>
<td>New CML/CP</td>
<td>45</td>
</tr>
<tr>
<td>New CML/CP</td>
<td>2</td>
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<tr>
<td>New CML in transformation</td>
<td>1</td>
</tr>
<tr>
<td>New CML/CP</td>
<td>4</td>
</tr>
<tr>
<td>Complex translocation</td>
<td>1</td>
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<tr>
<td>CML on interferon</td>
<td>5</td>
</tr>
<tr>
<td>TOTAL</td>
<td>58</td>
</tr>
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</table>

CML: Chronic myeloid leukemia
CP: Chronic phase
NM: No metaphase
Ph: Philadelphia chromosome
higher sensitivity rate of detection. A comparison with cytogenetics is shown in Table 3.

**PML/RARα.** Using the RT-PCR, 9 cases of acute promyelocytic leukemia studied showed the presence of the PML/RARα gene. Three cases showed the short isoform and 6 cases demonstrated high molecular weight bands of the long isoform. Figure 4 shows an example of the acute promyelocytic leukemia cases on lane 2 with the short isoform of 220 bp band and on lane 3 with the long isoform of 550 and 694 bp bands.

**BCL-2.** A total of 38 cases of non-Hodgkin’s lymphoma (NHL) were submitted for the detection of Bcl-2 gene re-arrangement. Twenty two cases with morphologic and immunophenotypic diagnosis of nodal follicular lymphoma (FL) were included. All lymph nodes were formalin-fixed and paraffin embedded tissues. Re-arrangements of the major breakpoint region (MBR) were evident in 8 out of 22 cases (36%) of FL determined by visualization of a discrete band of approximately 200 base pair. Figure 5 shows the results of 5 cases of follicular lymphoma (lane 1-5) with different size and intensity of approximately 200 bp. The technique is not quantitative, therefore, the intensity of the band reflects DNA amount and quality.

Rearrangement of the minor cluster sequence (MCS) of the Bcl-2 gene was examined in all 22 cases of FL. However, none demonstrated amplifiable PCR products.

The remaining 16 negative cases for MBR and MCS Bcl-2 gene re-arrangement included diffuse large cell lymphoma, lymphoblastic lymphoma, chronic lymphocytic leukemia, MAL T and Mantle zone lymphomas and lymphoid hyperplasia.

**Discussion.** Immunoglobulin and T-cell receptor gene re-arrangements, chromosomal translocations and oncogene disregulations have been traditionally evaluated with standard Southern blot (SB) methodology. Re-arrangements of immunoglobulin genes can be detected by SB in nearly all B-cell lymphomas. To detect re-arrangement, DNA is extracted from clinical samples, is cut with restriction endonuclease enzymes (EcoRI, BamHI and Hind III) and the digested fragments are electrophoresed through agarose gels and transferred to filter paper that is then hybridized with a radiolabeled probe. In its non re-arranged (germinal) configuration, the
probe would hybridize to a several kilobase DNA restriction fragment. However, in the case of a B-cell lymphoma, the probe will hybridize to an altered (smaller) restriction fragment that represents a clonal gene re-arrangement. Chronic lymphocytic leukemia, hairy cell leukemia, small cell and large cell lymphoma and follicular lymphoma show rearrangement of both heavy and light chain genes.\(^{10,17-20}\) In a similar manner, the T-cell receptor beta chain gene is re-arranged in the majority of peripheral T-cell lymphomas (mycosis fungoides, Sezary syndrome, diffuse mixed, large cell lymphomas, adult T-cell lymphoma/leukemia, angiocentric immunoproliferative lesions, angioimmunoblastic lymphadenopathy and Lennert's lymphoma).\(^{11,21-23}\)

The disadvantages of SB analysis (lengthy procedure, low sensitivity threshold, requirement of radioactivity and fresh/frozen tissues) have prompted investigators to develop alternative molecular diagnostic approaches. The discovery of polymerase chain reaction (PCR) has revolutionized molecular genetic diagnostics, with its primary value being its ability to enormously amplify target DNA or RNA.

PCR analysis has many advantages over conventional SB including the rapidity of the assay, the amenability to automation, no need for radioactivity, the need for very small amounts of starting DNA and its sensitivity. Using a single pair of primers to the FR3 region and the JH, we were able to detect 102 of 127 (80.3%) morphologically and immunophenotypically proven B-cell lymphoproliferative disease. This compares well with other laboratories using the same or similar primer pairs.\(^{10,18,20,24-26}\)

Amplification of rearranged T-cell receptor gamma chain genes (TCR gamma) by PCR for the detection of clonal T-cell population has been described in the literature.\(^{11,21-23}\) We have used primer mixes covering all possible TCR gamma recombinations in T-cell clones of T-cell acute lymphoblastic leukemia, and T-cell lymphoma samples aiming to a high detection rate compared to a single pair of primers. Those consensus TCR gamma chain V & J primers included V-1-8, V-9, and V-10, 11, 12. Using this approach, we were able to detect T-cell clonality in 91.7% of T-cell acute lymphoblastic leukemia.

The usefulness of this PCR approach was also tested on a 23 cases of clinically suspected cutaneous T-cell lymphoma (CTCL). Monoclonal TCR gamma re-arrangement was detected in 17 cases of morphologically proven CTCL and three cases of early stage disease.

Our false negative results (13%) are slightly higher in comparison to published range of 5-10%.\(^{27,28}\) Therefore, due to the difficulty in detecting T-cell monoclonality by immunohistochemical techniques, PCR can be used as a complementary technique in the routine diagnosis of CTCL.

Geographical or racial differences in the incidence of non-Hodgkin's lymphoma and, in particular, follicular lymphoma as well as the detection rate of Bcl-2 gene re-arrangement have been reported in the literature.\(^{29,34}\) The prevalence of follicular lymphoma in Saudi Arabia is less than 10% of all non-Hodgkin's lymphoma with diffuse large cell lymphoma being the most common histologic type at the time of presentation.\(^{35}\) Similar low incidence of follicular lymphoma and Bcl-2 gene re-arrangement is also reported from Japan.\(^{30,33}\)

Using primers for the Bcl-2 gene, we were able to detect re-arrangements in 8 out of 22 cases (36%) of follicular lymphoma in formalin-fixed paraffin embedded lymph node tissues hybridized with specific probe and detected by chemiluminescence technique. Although the number of cases is small compared to Western studies, the rate of detection in our study falls within the range reported by others for PCR analysis using DNA from fixed material.\(^{36,38}\)

Two major isoforms of PML and RARalpha fusion gene can be produced as a result of the t(15;17) in Acute Promyelocytic Leukemia (APL) depending on the location of the PML break point. Break points located within PML introns 3 or 4 (bcr3) are associated consistently with the production of short (S) type fusion mRNA between PML exon 3 (p3) and RARalpha exon 3 (r3), whereas those located within or downstream of PML intron 6 (bcr1) are associated consistently with formation of long (L) type chimeras between PML exon 6 (p6) and RARalpha exon 3 (r3).\(^{39,41}\)

The detection of t(15;17) and/or the PML-RARalpha gene is essential in the diagnosis of APL. The traditional cytogenetic methods, although very specific, are time-consuming and more laborious leading to delay of management in cases sometimes accompanied by serious abnormal coagulopathy such as disseminated intravascular coagulation. The detection of the PML-RARalpha gene by RT-PCR and/or FISH techniques is faster and more reliable. Also the detection of different PML-RARalpha gene isoforms could be of value in the management of M3 patients and in monitoring their response to the treatment by ATRA induction therapy.\(^{16}\)

The standard Ph chromosome is found in approximately 90% of cases of CML and cytogenetic variants account for a further 5%. Of the remaining 5%, roughly half show a BCR/ABL gene rearrangement by molecular studies.\(^{42}\)

Culture failure and inadequacy of sample also contributes to the low level of Ph chromosome detection rate by conventional cytogenetic methods. A single pair primer PCR has been used to amplify the BCR/ABL cDNA after reverse transcription of the mRNA. However, due to low sensitivity of the
technique leading to false negative results in the cases on interferon therapy, nested primer, strategy was used to detect minimal residual disease after therapy with interferone or post bone marrow transplantation.4-45

The application of PCR in diagnostic oncology has enormous potential for complementing morphology and flow cytometry and promises to serve as a DNA/RNA marker to assist in the diagnosis, staging and monitoring of lymphoma and leukemia and the detection of minimal residual disease after bone marrow transplantation.

References


752 Saudi Medical Journal 1998; Vol. 19 (6)